ORIGINAL ARTICLE

The immunomodulatory activity of the extracts and complexes of biologically active compounds of *Galium verum* L. herb

Imunomodulační aktivita extraktů a komplexů biologicky aktivních látek *Galium verum* L. herba

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Summary

The present article discusses the results of an immunomodulatory activity of Galii veri herba, *Galium verum* herb (*Galium verum* L., Rubiaceae) fluid water extract and the effect of the polysaccharide and phenolic complexes of the letter on the immunomodulatory activity. The same extract was fractionated into a polysaccharide complex (PSC) and a polyphenolic complex (PPC). In the obtained substances, the contents of hydroxycinnamic derivates, flavonoids and polyphenols were determined spectrophotometrically; polysaccharides were quantified gravimetrically; the immunomodulatory activity of the substances was determined in the reaction of lymphocyte blast transformation. It has been established that the fluid extract of *Galium verum* herb contains 6.3% polysaccharides, 4.2% hydroxycinnamic derivates expressed as chlorogenic acid, 0.4% flavonoids expressed as rutin, 3.7% polyphenols expressed as gallic acid. PPC contains 4.48% hydroxycinnamic derivates expressed as chlorogenic acid, 0.43% flavonoids expressed as rutin, and 3.95% polyphenols expressed as gallic acid. The lowest immunomodulatory activity was found for PPC. A significantly higher activity was determined for PSC. The highest immunomodulatory activity was established for the fluid extract at a dilution of 1/20, its activity being by 59.4% higher compared with the lymphocyte spontaneous transformation and by 18.5% higher than that of the reference substance PHA. The obtained results enable an assumption of a synergistic effect of PPC and PSC of *Galium verum* herb fluid extract on the potency of its immunomodulatory activity.

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Key words: *Galium verum* L. • immunomodulatory activity • lymphocyte blast transformation • fluid water extract • polysaccharide complex • polyphenols complex

Souhrn

Tento článek popisuje výsledky imunomodulační aktivity tekutého vodného extraktu získaného z natě svízele syřišťového, *Galium verum* herb (*Galium verum* L., Rubiaceae) a vliv polysacharidového a fenolického komplexu na imunomodulační aktivitu. Stejný extrakt byl frakcionován na polysacharidový komplex (PSC) a polyfenolický komplex (PPC). V získaných látkách byl spektrofotometricky stanoven obsah hydroxycinnamových derivátů, flavonoidů a polyfenolů; polysacharidy byly kvantifikovány gravimetricky; imunomodulační aktivita látek byla určena reakcí blastické transformace lymfocytů. Bylo zjištěno, že tekutý extrakt získaný z natě *Galium verum* obsahuje 6,3 % polysacharidů, 4,2 % hydroxycinnamových derivátů vyjádřeno jako kyselina chlorogenová, 0,4 % flavonoidů vyjádřeno jako rutin a 3,7 % polyfenolů vyjádřeno jako kyselina galová. PPC obsahoval 4,48 % hydroxycinnamových derivátů vyjádřeno jako kyselina chlorogenová, 0,43 % flavonoidů a 3,95 % polyfenolických sloučenin vyjádřeno jako kyselina galová. Pro PPC byla stanovena nejnižší imunomodulační aktivita. Výrazně vyšší aktivita byla stanovena pro PSC. Nejvyšší imunomodulační aktivita byla stanovena pro tekutý extrakt při zředění 1/20, jehož aktivita byla o 59,4 % vyšší ve srovnání se spontánní transformací lymfocytů a o 18,5 % vyšší než aktivita referenční látky PHA. Ze získaných výsledků lze předpokládat synergický účinek PPC a PSC tekutého extraktu z natě *Galium verum* na účinnost jeho imunomodulační aktivity.

Klíčová slova: *Galium verum* L. • imunomodulační aktivita • blastická transformace lymfocytů • tekutý vodný extrakt • polysacharidový komplex • polyfenolický komplex

Introduction

The secondary immunodeficiency disorders present a severe challenge for the modern society. The use of immunosuppressive drugs, chemotherapy, malnutrition,

chronic diseases and metabolic disorders are among root causes of secondary immunodeficiencies[1]. Since all these conditions lead to serious biochemical disturbances affecting different organ systems, correction of these changes requires a complex and long-term treatment. Considering the above-mentioned, a search for immunomodulators of plant origin is among the topical issues facing pharmacy[2–4]. Herbal medicinal products uniquely combine a low toxicity, an affinity to the human body and a wide range of biological activities (antioxidant, anti-inflammatory, immunostimulating, antiviral, antimicrobial, etc.) causing a multifaceted beneficial effect on the impaired body functions, which is of great importance in the treatment of the secondary immunodeficiencies[5–9]. On the pharmaceutical market of Ukraine, the line of immunomodulators of plant origin is rather limited. Therefore, it is currently important for medicine and pharmacy to seek for and develop effective domestic herbal medicinal products with an immunomodulatory effect. In Ukrainian folk medicine Galii veri herba, *Galium verum* herb (*Galium verum* L., Rubiaceae) is used as antibacterial, haemostatic, choleretic and immunostimulant agent[10]. The herb of *Galium verum* contains iridoids, flavonoids, coumarins etc. The aim of the present article is to discuss the effect of the fluid water extract of *Galium verum* herb, polysaccharide complex (PSC) and polyphenolic complex (PPC) on the functional activity of lymphocytes in the reaction of lymphocyte blast transformation (RLBT).

Experimental part

Plant materials

Galium verum herb was harvested at full flowering stage in the Kharkiv region (Ukraine) in the summer of 2016. Herbarium samples (No. 20062016-25062016) are deposited at the Department of Pharmacognosy (National University of Pharmacy, Ukraine).

Preparation of extracts

A three-fold water extraction (30 min each) was carried out at a general ratio of the plant material : solvent of 1 : 10 on heating. Three extracts were combined, concentrated on a vacuum rotary evaporator to a ratio of plant material – finished product of 1 : 1.

At the same time, under the same conditions, an extract was obtained from which polysaccharides were precipitated with three volumes of 96% ethanol, separated by centrifugation (10 min, 3000 rpm), re-washed with 96% ethanol and centrifuged under the same conditions. The resulting polysaccharide complex was dried to an air-dry state (PSC). The filtrate remaining after polysaccharides precipitation was concentrated to a ratio of plant material – finished product of 1 : 1 (PPC – polyphenolic complex).

Preliminary phytochemical screening of Galium verum herb fluid water extract, PSC and PPC

The preliminary phytochemical screening was performed to identify the main groups of biologically active compounds (BAC) of *Galium verum* L. herb fluid water extract, PSC and PPC, namely sugars, flavonoids, polyphenols by a reaction with Fehling's solution, by a cyanidin reaction, and a reaction with lead acetate and ferric chloride.

Quantification of main groups of BACs

Polysaccharides were quantified gravimetrically after complete drying at room temperature taking into account the loss on drying[11, 12]. PSC's monosaccharides after acidic hydrolysis were quantified by the spectrometric method using the reaction with picric acid (as glucose, λ = 463 nm) according to the procedures developed by Minin et al.[13]. In *Galium verum* L. herb fluid water extract and PPC, the content of hydroxycinnamic derivates was determined by direct spectrophotometry (as chlorogenic acid, λ = 325 nm) according to Yezerska et al.[14], Spagnol et al.[15]; flavonoids were quantified by the method of differential spectrophotometry with aluminium chloride (as rutin, λ = 410 nm)[16]; polyphenols were quantified by direct spectrophotometry (as gallic acid, λ = 270 nm) according to Kovalyova et al.[17], Koshovyi et al.[18]. All assays were performed in triplicate.

Study of immunomodulatory activity

The study of the immunomodulatory activity of PSC was carried out at the dose which corresponds to polysaccharide content in the fluid water extract, i.e. 0.63 g of PSC were dissolved in 10 mL of purified water. To study the immunomodulatory activity of *Galium verum* L. herb fluid water extract and PPC, 10 mL of each substance were used. Before the RLBT, the fluid water extract, PPC and solution of polysaccharides were prepared in ratios of 1/200, 1/20, 1/10. 100 µL of substances were added to 100 µL of primary cultures of immunocompetent cells.

The immunomodulatory activity of the substances was studied *in vitro* in a reaction of lymphocyte blast transformation (RLBT)[19, 20].

As the sample for substance testing, the mononuclear cells (lymphocytes) removed from venous heparinized blood (donated blood, Kharkiv Regional Blood Banking Centre, UA) by ficoll-verographine gradient density centrifugation (density 1.077 g/mL) (Research and Production Enterprise "PanEco", RU) by the standard technique[21], were used (Protocol of the Committee on Biomedical Ethics of SO "Mechnikov Institute of Microbiology and Immunology" No. 2 of May 16, 2017).

The cells obtained were cultured in medium 199 with an addition of 10% bovine foetal serum (Thermo Fisher Scientific, BR), 2 mM L-glutamine (Altera Holding, RU), 100 µg/mL gentamicin (LEK (CZ). A suspension of 1 million cells per 1mL of the culture medium with the addition of substances was incubated for 15–18 hours in a thermostat at 37 °C, in a 5% CO_2 atmosphere with saturated water vapour.

The intensity of the proliferative reaction was evaluated by the indexes of DNA synthesis activation recorded by the treatment of samples with antiBrdU Antibody (3H579) monoclonal antibodies (Santa Cruz Biotechnology, USA) at the concentration of 100 mg/mL. After the final sample preparation, numerical data on the total number of

cells and the percentage of blast forms in the samples were established for a flow cytometric analysis with fluorescence detection.

It is known that vegetable lectin phytohemagglutinin (PHA) (Research and Production Enterprise "PanEco", RU) is a mitogen for all T-lymphocytes[22]. Therefore, in order to determine the quality of the cell cultivating environment, as well as to study the potential proliferative activity of the major populations of T-lymphocytes (donated blood, Kharkiv Regional Blood Banking Centre, UA), the mitogenic stimulation of lymphocytes by PHA at a concentration of 2.5 µg/mL was performed as the control. RLBT without the addition of the substances under study (spontaneous blast transformation) was also evaluated.

Equipment

A spectrophotometer Evolution™ 60S UV-Visible (Thermo Fisher Scientific, USA), electronic analytical scales AN 100 "Axis" (AXIS, PL), an electrical temperature chamber TC80M-3 (Medlabortekhnika, UA), a centrifuge OPN – 3 (Phizpribor, RU), a microscope ZEISS Primo Star (ZEISS, DE), a pipette Thermo Scientific, Lait series 1–200 µl (Thermo Fisher Scientific, USA), a pipette Thermo Scientific, Lait series 1–50 µl (Thermo Fisher Scientific, USA), a pipette Thermo Scientific, Lait series 1–1000 µl (Thermo Fisher Scientific, USA), a pipette Thermo Scientific, Lait series 1–20 µl (Thermo Fisher Scientific, USA), a CO_2 incubator (Binder, DE), a bioanalyzer Agilent 2100 (Agilent, DE).

Chemicals

The purified water used for the extraction complied with the requirements of the State Pharmacopoeia of Ukraine[23]; chemicals used for phytochemical screening and quantification of the main groups of BACs: ethanol (ACS reagent, Fisher Scientific, USA), hydrochloric acid, p.a. (Sobstar, Zaporizhia, UA), acetic acid, puriss. (PJSC AZOT, UA), lead (II) acetate, p.a. (Unikhim Ltd., RU), aluminum chloride, p.a., granulated zinc, p.a. (PC Uralskiy zavod khimicheskih reaktivov, RU), ferric (III) chloride, puriss. (Sigma-Aldrich, USA), Fehling's solutions (Biochem Chemopharma, FR), picric acid, p.a. (Sigma-Aldrich, USA), gallic acid, chlorogenic acid and rutin were of analytical grade (Merck, DE).

Statistical analysis

All statistical analyses were performed using Microsoft Office Excel 2007[24]. Differences between groups were statistically analysed using one-way analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD). P values less than 0.05 were considered statistically significant.

Result and discussion

Phytochemical screening of Galium verum herb fluid water extract, PSC and PPC

The fluid water extract, polysaccharide complex and polyphenolic complex of *Galium verum* herb were obtained for the first time and their phytochemical profiles were characterized for the first time too.

The phytochemical screening of *G. verum* herb fluid water extract revealed the presence of reducing sugars, flavonoids, polyphenols. The results obtained correspond with previous studies aimed at the research of phytochemical constituencies of *Galium* species[25–27].

Quantification of main groups of BACs

When quantifying the main groups of BACs, it was established that the fluid water extract contained 6.3% polysaccharides, 4.2% hydroxycinnamic derivates expressed as chlorogenic acid, 0.4% flavonoids expressed as rutin, 3.7% polyphenols expressed as gallic acid. PPC contains 4.48% hydroxycinnamic derivates expressed as chlorogenic acid, 0.43% flavonoids expressed as rutin, 3.95% polyphenols expressed as gallic acid. In PSC the content of monosaccharides was 26.70%; taking into account the loss on drying (7.2%), the total ash was 23.61%.

Since the fluid extract was obtained at the ratio of the plant material – finished product 1 : 1, we could compare the content of BACs in the fluid extract with the content of BACs in the plant material.

There are no available articles discussing the phytochemical profile of water extracts of *Galium* species, but in comparison with the data provided by Vlase L, Mocan A, Hanganu D, et al.[25] and taking into account the differences in the extracts used, the content of hydroxycinnamic derivates and polyphenols in the fluid extract and PPC is higher, and the content of flavonoids is significantly lower.

Reaction of lymphocyte blast transformation in vitro

The immunomodulatory activity of *Galium verum* herb fluid water extract and the effect of the polysaccharide and phenolic compounds of the latter on the immunomodulatory activity were studied for the first time.

It has been established that all the substances under study greatly stimulate the transformational activity of peripheral blood mononuclear cells. The activity under the influence of substances increases from 26.6% (PPC at dilution of 1/200) to 59.4% (fluid extract at dilution of 1/20) compared with spontaneous lymphocyte blast transformation. Table 1 shows the effect of substances of *Galium verum* L. herb on the indices of lymphocyte blast transformation.

The highest activity was found for *Galium verum* water fluid extract at a dilution of 1/20, its activity being by 59.4% higher than that of the lymphocyte spontaneous transformation and by 18.5% higher than that of the reference substance PHA. Somewhat lower indexes were found for the extract at a dilution of 1/200, its activity being by 54.4% higher than that of the lymphocyte spontaneous transformation and by 13.5% higher than that of PHA; the lowest activity was exhibited at a dilution of 1/10, its activity being by 53.1% higher than that of the lymphocyte spontaneous transformation and by 11.1% higher than that of PHA.

Table 1. The effect of substances of Galium verum L. on the indices of lymphocyte blast transformation (X ± m), n = 5

Substances	Dilution of the extract	RLBT (%)
Extract	1/200	62.7 ± 2.2*
	1/20	67.7 ± 2.6*
	1/10	61.3 ± 2.4*
PPC	1/200	34.9 ± 3.7
	1/20	45.6 ± 3.3
	1/10	45.0 ± 3.4
PSC	1/200	49.0 ± 3.2
	1/20	61.7 ± 2.3*
	1/10	59.4 ± 3.3*
Control (PHA)	–	49.2 ± 2.3
Spontaneous RLBT	–	8.3 ± 0.6

PPC – polyphenolic complex, PSC – polysaccharide complex, PHA – phytohemagglutinin, RLBT – the reaction of lymphocyte blast transformation
*$P < 0.05$ in comparison with the indices of control

The water solution of PSC showed a significant activity at a dilution of 1/10 (its activity was by 51.1% higher than that of the lymphocyte spontaneous transformation and by 10.2% higher than that of PHA), at a dilution of 1/20 its activity being by 53.4% higher than that of the lymphocyte spontaneous transformation and by 12.5% higher than that of PHA, at a dilution of 1/200 its activity being by 40.7% higher than that of the lymphocyte spontaneous transformation and almost comparable with PHA activity.
The lowest activity was found for PPC: at a dilution of 1/10 its activity was by 36.7% higher than that of the lymphocyte spontaneous transformation and by 4.2% lower than that of PHA; at a dilution of 1/20 its activity was by 37.3% higher than that of the lymphocyte spontaneous transformation and by 3.6% lower than that of PHA; at a dilution of 1/200 its activity was by 26.6% higher than that of the lymphocyte spontaneous transformation and by 14.3% lower than that of PHA.
In our previous study, we reported the immunomodulatory effects of two *Asperula* species (Rubiaceae family) which are native herbs used for the treatment of inflammatory diseases in Ukrainian traditional medicine[28].
According to research articles available in the public domain, only for one species of the genus – *Galium mite* – the immunomodulatory activity was studied [29]. It was concluded that the methanol extract of the aerial part of *G. mite* mildly increases the proliferation of human lymphocytes at the concentrations of less than 10 μg/ml and inhibits their proliferation at higher doses in a dose-dependent manner. As the results of our study have shown, all the substances of *Galium verum* L. herb and especially fluid water extract are characterized by a stimulating effect on the transformational activity of human lymphocytes. We can assume that different effects of *G. verum* L. herb water extract and methanol extract of aerial part of *G. mite* on human lymphocyte proliferation can be explained by differences in the phytochemical profiles of species and extracts under study.
PHA increased the level of RLBT by 40.9% compared with the lymphocyte spontaneous transformation.
On average, the most potent stimulation of immunocompetent cell functional activity was observed in 100 μL of the substances under study at a dilution of 1/20.
The obtained results suggested a synergistic effect of the polysaccharide complex and the phenolic complex of *Galium verum* herb fluid water extract on the potency of immunomodulating activity.
The obtained data provided the basis for an in-depth study of the mechanisms of immunomodulatory activity of the substances of *Galium verum* herb in order to develop on their basis medicinal products for immune correction of the conditions occurring in violation of the cooperative function of immunocompetent cells and the correction of weakening of nonspecific resistance factors.

Conclusion

Fluid water extracts of *Galium verum* herb were obtained for the first time; their chemical composition and immunomodulatory activity were studied. *Galium verum* herb fluid extract and polysaccharide complex greatly stimulate the transformational activity of blood immunocompetent cells. The obtained results confirmed the prospect of further study of substances of *Galium verum* in order to search for new drugs and principles of immunodeficiency correction.

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